Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus

Larry S. Barak, Robert H. Oakley, Stéphane A. Laporte, and Marc G. Caron*

Howard Hughes Medical Institute Laboratories and Departments of Cell Biology and Medicine, Duke University Medical Center, Durham, NC 27710

Edited by Lutz Birnbaumer, University of California, Los Angeles, Los Angeles, CA, and approved November 1, 2000 (received for review June 30, 2000)

Agonist-dependent desensitization and internalization of G protein-coupled receptors (GPCR) are mediated by the binding of arrestins to phosphorylated receptors. The affinity of arrestins for the phosphorylated GPCR regulates the ability of the internalized receptor to be dephosphorylated and recycled back to the plasma membrane. In this study, we show that the naturally occurring loss of function vasopressin receptor mutation R137H, which is associated with familial nephrogenic diabetes insipidus, induces constitutive arrestin-mediated desensitization. In contrast to the wild-type vasopressin receptor, the nonsignaling R137H receptor is phosphorylated and sequestered in arrestin-associated intracellular vesicles even in the absence of agonist. Eliminating molecular determinants on the receptor that promote high affinity arrestin–receptor interaction reestablishes plasma membrane localization and the ability of the mutated receptors to signal. These findings suggest that unregulated desensitization can contribute to the etiology of a GPCR-based disease, implying that pharmacological targeting of GPCR desensitization may be therapeutically beneficial.

The G protein-coupled receptors (GPCRs) are plasma-membrane proteins that initiate intracellular signaling cascades by binding extracellular ligands (1, 2). GPCRs regulate many physiological processes, including vision, taste, cardiovascular function, and water balance, while sharing a number of common structural features. They contain seven transmembrane domains bridged by alternating intracellular and extracellular loops and an intracellular C-terminal of variable length. The intracellular loops bind the G proteins that activate effectors, which generate second messengers. Intracellular domains, particularly at the C termini, are phosphorylated by G protein-coupled receptor kinases (GRKs) in an agonist-dependent fashion (1, 2). Arrestin proteins then preferentially bind the GRK-phosphorylated receptors (3, 4), preventing them from activating G proteins (1).

The increased affinity of activated receptors for arrestins results in the observable translocation of arrestins from the cell cytosol to the plasma membrane, frequently followed by clathrin-coated pit internalization of the arrestin-bound receptors (5–9). Arrestin translocation has been visualized by using fusion proteins of green fluorescent protein (GFP) homologues and an arrestin family member (visual arrestin, βarrestin1 and βarrestin2) for numerous GPCRs including the angiotensin, neurokinin, thyrotropin-releasing hormone, and human vasopressin type II receptors (V2R) (5, 6, 10–12). These four receptors are representative of a larger class of GPCRs that form stable endocytic complexes with arrestins for extended periods (11). In particular, in the V2R, a cluster of GRK-phosphorylated serines in the C-tail regulates this high affinity interaction, which in turn influences the magnitude of V2R internalization and its rate of resensitization (11, 13).

An inability of the V2R to respond to arginine vasopressin (AVP) with the production of cAMP leads to a loss of the kidney's ability to concentrate urine and results in the water-losing syndrome nephrogenic diabetes insipidus (NDI; refs. 14 and 15). A variety of mutations in the V2R resulting in abnormal ligand binding, G protein coupling, and receptor trafficking have been associated with NDI (16–18). One of the first-characterized naturally occurring mutations of the V2R associated with NDI was a substitution of arginine 137 by histidine, V2R(R137H) (14, 18–20). Arginine 137 is found at the cytoplasmic end of transmembrane III in a highly conserved GPCR motif (DRY/H) (21). The assumed molecular basis for V2R(R137H) NDI is the inability of ligand-bound receptor to activate G proteins (19, 20). However, an analogous mutation engineered in the equivalent ERY motif of rhodopsin (R135A or L) resulted in increased rhodopsin affinity for rhodopsin kinase and visual arrestin (22). Therefore, we hypothesized that the apparent loss of function in the V2R(R137H) mutant might result from its increased interaction with arrestins. In this study, we demonstrate that the V2R(R137H) interacts with βarrestins in the absence of agonist and that this constitutive interaction underlies its phenotypic properties. These findings provide an example of a naturally occurring GPCR signaling defect that can be attributed to a constitutively desensitized receptor.

Experimental Procedures

Materials. Arginine vasopressin was obtained from Sigma, and [^{3}H]AVP was from Amersham Pharmacia. HEK-293 cells were from the American Type Culture Collection, and cell culture reagents were from Life Technologies (Rockville, MD) and Cellco (Kensington, MD).

Plasmids and Constructs. The N-terminal hemagglutinin (HA)-tagged V2R, the V2R(Ala6), and the V2R(T362) were expressed in pcDNA3.1/zeo (11), and their R137H analogues were expressed in pEGFP-N3 (CLONTECH) with stop codons intact using *Sac*I/*Sal*I restriction sites. The GFP conjugates of the V2R and V2R(R137H) were generated by PCR and inserted in frame at the *Xho*I/*Sal*I and *Sac*I/*Sal*I restriction sites of pEGFP-N3, respectively. βarrestin2-GFP (S65T) was constructed as described (23).

Cell Culture and Transfection. HEK-293 cells were grown in Eagle's MEM with Earle's salt (MEM) supplemented with 10% (vol/vol) FBS and a 1:100 dilution of penicillin/streptomycin (Sigma). Cells were transiently transfected with plasmid cDNA using a modified calcium phosphate coprecipitation method (24).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AVP, arginine vasopressin; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; NDI, nephrogenic diabetes insipidus; V2R, human vasopressin receptor; HA, hemagglutinin.

*To whom reprint requests should be addressed at: Howard Hughes Medical Institute Laboratories and Departments of Cell Biology and Medicine, Duke University Medical Center, Box 3287, Durham, NC 27710. E-mail: caron002@mc.duke.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.011303698. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.011303698

BIOCHEMISTRY

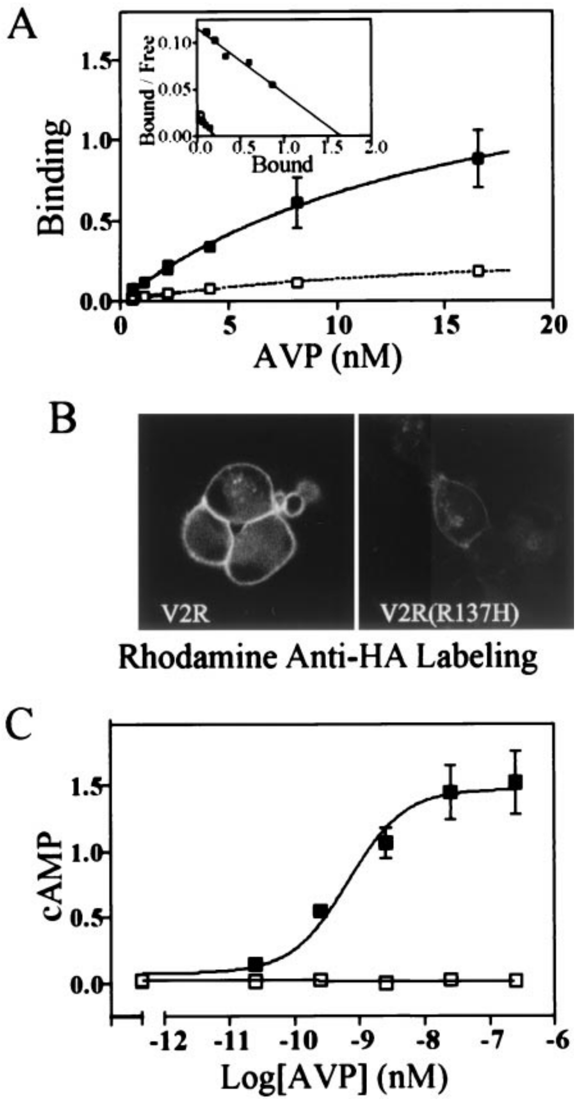

Fig. 1. Expression and adenylyl cyclase stimulation of V2R and V2R(R137H) in HEK-293 cells. Cells transiently transfected with cDNA for V2R (■) or V2R(R137H) (□) were exposed to varying concentrations of [^{3}H]AVP. (*A*) Scatchard analysis (*Inset*) indicates the receptors have similar affinity for AVP [V2R, 16 ± 6 nM; V2R(R137H), 15 ± 3 nM]. V2R expression varied between 2.5 and 5 pmol/mg of cell protein, with the plasma membrane expression of the V2R(R137H) being approximately 1/12 of this (*x* intercept of Scatchard). The data are representative of three experiments, with each point measured in duplicate. (*B*) Fluorescence images of live unpermeabilized cells, labeled with rhodamine-tagged mouse-monoclonal anti-HA antibodies, expressing the V2R (*Left*) or the V2R(R137H) (*Right*). (*C*). cAMP measured in whole cells stimulated for 15 min with concentrations of AVP between 0 and 250 nM. cAMP accumulation is expressed as total counts of [^{3}H]cAMP/total uptake of [^{3}H]adenine per well of cells. Results are the mean ± SD of three experiments.

Receptor Binding and Adenylyl Cyclase Assays. HEK-293 cells plated into 12-well Falcon dishes were washed twice in cold MEM, incubated for 30 min with a 250-μl solution of 2% BSA in 4°C MEM containing [^{3}H]AVP, and washed three times with cold MEM/BSA; then, the cell-bound [^{3}H]AVP was extracted with 250 μl of 0.5 M NaOH in PBS, neutralized with HCl, and measured using a liquid scintillation counter. Nonspecific binding was determined in the presence of a 100-fold excess of cold AVP. cAMP production in intact HEK-293 cells containing V2R variants was measured as described (24).

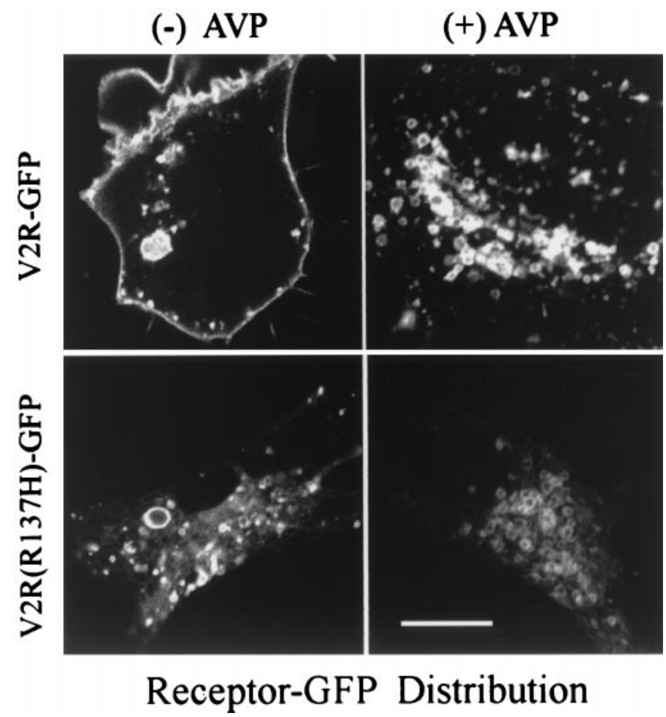

Fig. 2. Fluorescence images of V2R-GFP and V2R(R137H)-GFP in HEK-293 cells. Cells expressing V2R-GFP (*Upper*) or V2R(R137H)-GFP (*Lower*) were treated with vehicle or AVP for 30 min at 37°C. The agonist-mediated redistribution of wild-type receptor (*Upper*) from the plasma membrane to endocytic vesicles contrasts with the agonist-independent localization of the V2R(R137H)-GFP in endosomes (*Lower*). Bar = 25 μM.

Confocal Microscopy. HEK-293 cells were plated on the day following transfection onto collagen-treated 35-mm glass-bottom culture dishes (Sigma). Confocal microscopy was performed with a Zeiss laser-scanning microscope (LSM-510). GFP images were collected using 488-nm argon excitation and a 505-nm long-pass filter.

Antibody Labeling. Live cells were incubated at room temperature with a 1:100 dilution of rhodamine-tagged anti-HA mouse monoclonal antibody (Boehringer Mannheim) in a 2% BSA/MEM solution with 10 mM Hepes for 40 min and then washed three times in MEM/Hepes.

Whole Cell Phosphorylation. Receptor phosphorylation was performed essentially as described (11). Equivalent amounts of receptor in each sample, as determined by [^{3}H]AVP binding on whole cells, were subjected to SDS/PAGE.

Receptor Immunoprecipitation and Western Blotting. HEK-293 cells were stimulated with or without AVP for 10 min, washed with ice-cold PBS, scraped into precipitation buffer (11), and solubilized for 1 h at 4°C. After centrifugation, supernatants were collected, and HA-tagged receptors were immunoprecipitated at 4°C using the anti-HA 12CA5 mouse monoclonal antibody (Boehringer Mannheim). Recovered proteins were subjected to SDS/PAGE and immunoblotted with the anti-HA rabbit polyclonal antibody (Babco, Richmond, CA).

Results

Expression and Signaling of V2R and V2R(R137H) in HEK-293 Cells. The whole cell binding data in Fig. 1*A* indicate that the V2R and V2R(R137H) have the same affinity for AVP. In contrast, the Scatchard plot (*Inset*) suggests that plasma membrane

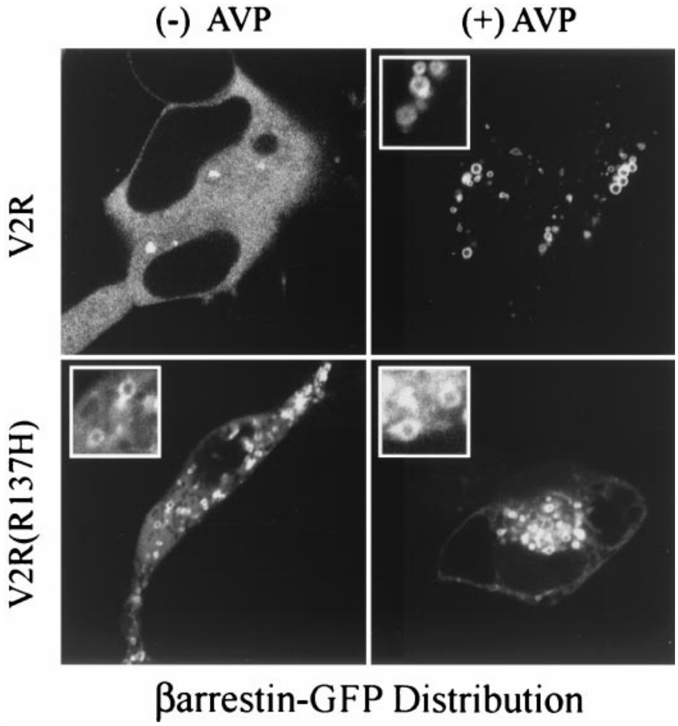

Fig. 3. Fluorescence images of the association between βarrestin2-GFP and V2R or V2R(R137H) in HEK-293 cells. *Left* shows the agonist-independent distribution of βarrestin2-GFP in living cells expressing the V2R (*Upper*) or V2R(R137H) (*Lower*). Without agonist treatment, βarrestin2-GFP is cytosolic in cells containing wild-type V2R (*Left Upper*), but in cells containing the V2R(R137H) (*Left Lower*) it is also in endocytic vesicles. Following 30 min of exposure to 100 nM AVP (*Right*), βarrestin-GFP is localized on endocytic vesicles with both receptor subtypes.

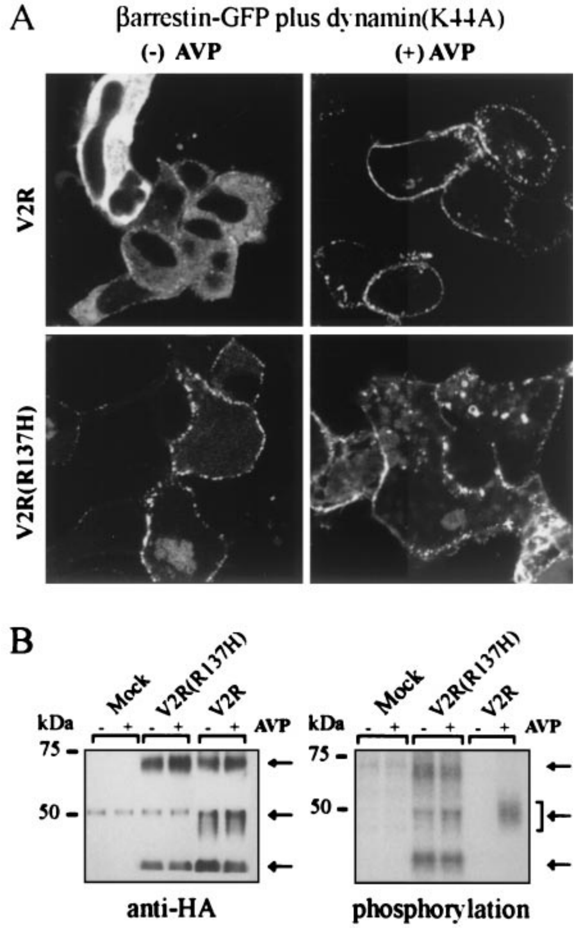

Fig. 4. βarrestin2 association with and phosphorylation of V2R and V2R(R137H) in HEK-293 cells. (*A*) Dynamin(K44A) was expressed with βarrestin2-GFP and either V2R or V2R(R137H). Exposure of V2R to AVP (100 nM) (*Upper Right*) resulted in appreciable βarrestin2-GFP translocation that remains visible at 30 min as a punctate distribution at the cell membrane rather than as a vesicular distribution inside the cell. In the absence of agonist, the cells containing dynamin(K44A) and V2R(R137H) (*Lower Left*) also show βarrestin2-GFP fluorescence distributed in punctate areas at the plasma membrane. A similar pattern was apparent even in the presence of agonist (*Lower Right*). (*B*) *Left* shows receptors that were immunoprecipitated with a mouse anti-HA antibody and blotted with a rabbit anti-HA antibody. The faint 50-kDa band present in all six lanes is cross-reactive mouse Ig heavy chain. *Right* shows depicts receptors that were assayed for phosphorylation as described in *Experimental Procedures*. Equal amounts of receptor (40 fmol) were loaded into each lane. The arrows mark the positions of the receptor species migrating at approximately 70, 50, and 40 kDa as revealed by anti-HA antibody. Results are representative of three experiments.

V2R(R137H) expression is much lower. This finding is confirmed by comparing the amount of fluorescence originating from HA-tagged V2R (Fig. 1*B*, *Left*) and V2R(R137H) (*Right*) at the surface of live, unpermeabilized cells labeled with anti-HA antibody. Fig. 1*C* shows the amount of whole cell, AVP-stimulated (0–250 nM) cAMP production in cells transfected with V2R or V2R(R137H). Essentially, no adenylyl cyclase activity above basal is observed in the V2R(R137H) transfected cells. These data seemingly indicate, as do earlier studies (18, 20), that the V2R(R137H) NDI phenotype perhaps arises from an inability of the receptor to activate G protein.

Distribution and Trafficking of V2R and V2R(R137H) in Response to AVP. Many GPCRs express mutations that uncouple the receptors from G proteins without affecting receptor expression at the plasma membrane (20, 24, 25). Even though plasma membrane expression of the V2R(R137H) is relatively low, its intracellular complement in the absence of agonist is relatively abundant (20). Thus, the R137H mutation may also affect the trafficking mechanisms that determine receptor localization (16). To study V2R and V2R(R137H) trafficking in HEK-293 cells, we used a strategy using V2R- or V2R(R137H)-GFP chimeras (23, 26). Fig. 2 demonstrates that in the absence of agonist, V2R-GFP fluorescence originates predominantly from the plasma membrane (*Upper Left*). The addition of 100 nM AVP produces a loss of membrane fluorescence and a redistribution of V2R to endocytic vesicles (*Upper Right*) in a manner similar to wild-type V2R (11). Vesicles can be observed in either the cytosol or in the perinuclear region (27), depending on the time after agonist addition or the position of the confocal slice through the cell. In contrast, the majority of V2R(R137H)-GFP is cytosolic and vesicular (*Lower Left*) in the absence of agonist, and exposure to 100 nM AVP for 30 min does not appreciably alter V2R(R137H)-GFP distribution (*Lower Right*).

Distribution and Trafficking of βarrestin in HEK-293 Cells Expressing V2R or V2R(R137H) in Response to AVP. AVP-mediated endocytosis of the V2R in HEK-293 cells has been demonstrated to require βarrestins (11). The observed vesicular localization of the V2R(R137H)-GFP in the absence or presence of AVP is characteristic of βarrestin-mediated endocytosis and suggests that the V2R(R137H) may bind βarrestin sufficiently well without agonist to promote its own internalization. To examine the interaction of βarrestin with the V2R(R137H) in live HEK-293 cells, we transfected either the V2R or V2R(R137H) and a βarrestin2-GFP fusion protein (5, 11). When βarrestin2-GFP is

BIOCHEMISTRY

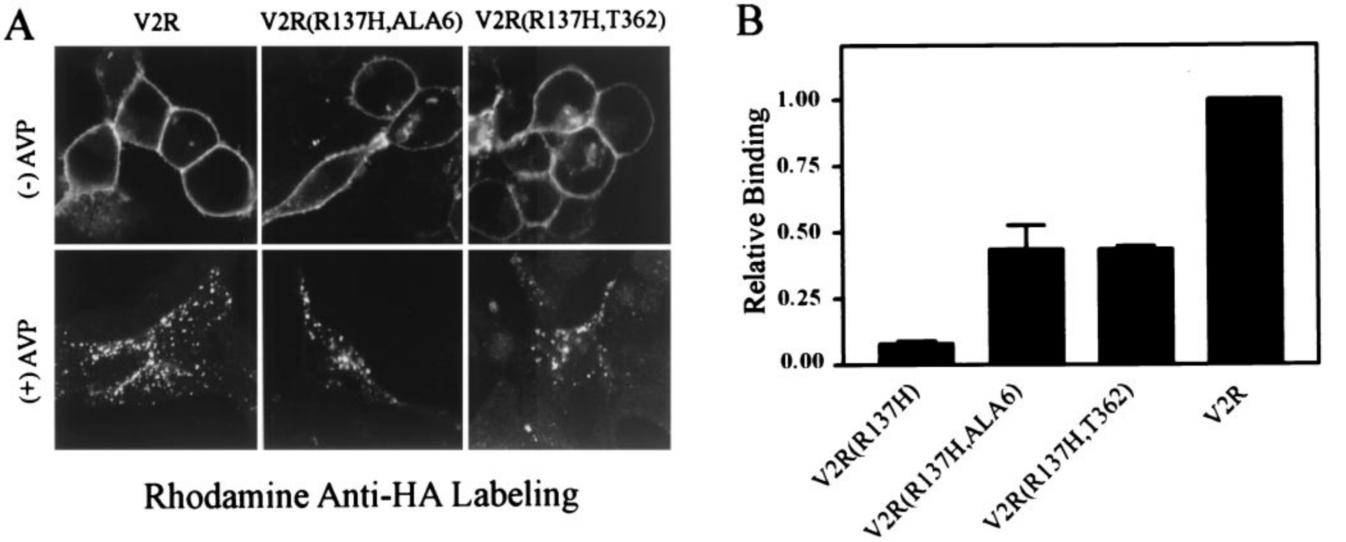

Fig. 5. Expression of V2R, V2R(R137H,Ala6), and V2R(R137H,T362) in HEK-293 cells. (*A*) Plasma membrane receptors were labeled with rhodamine-tagged mouse-monoclonal anti-HA antibody. The upper panels show receptor distribution in the absence of agonist. The lower panels show cells that were labeled with antibody before treatment with 100 nM AVP for 30 min at 37°C. (*B*) Plasma membrane receptor expression measured by [^{3}H]AVP was normalized to wild-type V2R plasma membrane expression (approximately 5 pmol/mg). Data are expressed as the mean ± SD from three independent experiments.

expressed with the V2R in the absence of agonist, the fluorescence is cytosolic and homogeneous (Fig. 3 *Upper Left*). Addition of AVP results in the translocation of βarrestin to plasma membrane V2R (11), the subsequent AP2-directed clustering of the βarrestin–receptor complex in clathrin-coated vesicles (28–30), and internalization of the βarrestin–receptor complex into endosomes (11). The appearance of βarrestin2-GFP in endosomes following AVP treatment is indicated by the vesicular distribution of GFP fluorescence shown in Fig. 3 (*Upper Right* and *Inset*). In contrast to our findings for the V2R, βarrestin2-GFP is distributed in endosomes in cells expressing the V2R(R137H) independent of agonist (Fig. 3 *Lower*). The localization of βarrestin-GFP in endocytic vesicles (*Insets*) suggests that the intracellular V2R(R137H) population may arise from plasma membrane receptors through a βarrestin-directed process.

Inhibition of Internalization of V2R or V2R(R137H)–βarrestin Complex in the Presence of Dominant Negative Dynamin. The cytosolic protein dynamin is required for the separation of clathrin-coated vesicles from the plasma membrane, and overexpression of the dynamin(K44A) variant, which competitively inhibits clathrin-coated vesicle dissociation, has been used to assess clathrin-mediated GPCR internalization (7). In HEK-293 cells in the absence of agonist, the expression of dynamin(K44A) with the V2R did not significantly change the homogeneous cytosolic distribution of βarrestin-GFP (Fig. 4*A*, *Upper Left*). In contrast, after 30 min of AVP treatment, βarrestin-GFP remained at the plasma membrane in a punctate distribution and did not traffic into endocytic vesicles (compare Fig. 4*A*, *Upper Right*, and Fig. 3). In the absence of agonist, the simultaneous expression of dynamin(K44A) and V2R(R137H) produced a plasma membrane distribution of βarrestin2-GFP (Fig. 4*A*, *Lower Left*) similar to the βarrestin2-GFP distribution observed for the V2R in the presence of agonist (Fig. 4*A*, *Upper Right*). AVP addition did not appreciably change the plasma membrane distribution of βarrestin2-GFP (Fig. 4*A*, *Lower Right*); however, βarrestin2–GFP-coated vesicles did become apparent in some cells. The ability of dynamin(K44A) to redistribute the complement of cytosolic βarrestin2-GFP to the plasma membrane in cells expressing the V2R(R137H) suggests that the intracellular mutant V2R(R137H), which is associated with βarrestin (Fig. 3 *Lower*), can traffic to the plasma membrane and that the spontaneous association of βarrestin with the receptor in the absence of agonist is sufficient to induce receptor internalization.

Constitutive Phosphorylation of V2R(R137H). The affinity between many GPCRs and βarrestin is regulated by GRK phosphorylation. Therefore, we examined the phosphorylation states of the V2R and V2R(R137H). Western blot analysis of immunoprecipitated V2R revealed three major species of this receptor migrating at approximately 70, 50, and 40 kDa (Fig. 4*B*, *Left*, arrows). The amount of basal phosphorylation observed for each V2R species was minimal, and only the 50-kDa form was phosphorylated in response to agonist (Fig. 4*B*, *Right*). Western blot analysis of immunoprecipitated V2R(R137H) revealed forms of this receptor migrating at approximately 70 and 40 kDa (Fig. 4*B*, *Left*) that were sensitive to digestion by Endoglycosidase H and PNGase A (data not shown), most probably representing immature glycosylated forms of the receptor (31, 32). Each of these forms of the V2R(R137H) was constitutively phosphorylated (Fig. 4*B*, *Right*). Although a 50-kDa species of the V2R(R137H) was not detected on the Western blot, the more sensitive phosphorylation assay revealed a small amount of agonist-mediated phosphorylation to this form of the receptor (Fig. 4*B*, *Right*, middle arrow), which is most probably membrane associated (31, 32). Moreover, the 50-kDa form of the V2R(R137H) was also phosphorylated in the absence of agonist, consistent with previous observations (19). Therefore, it appears that the abnormal phenotypic behavior of the V2R(R137H) may primarily reflect the constitutive association of βarrestin with a phosphorylated receptor. Whether βarrestin associates with the immature phosphorylated form of the mutated receptor is difficult to determine. However, the fact that βarrestin-GFP redistributes to the plasma membrane (as shown in Fig. 4) in the presence of the mutant dynamin suggests that βarrestin can bind preferentially forms of V2R capable of cellular trafficking.

Reversal of V2R(R137H) Phenotype and βarrestin Affinity. An increased receptor affinity for arrestins in the absence of agonist could mask the ability of the V2R(R137H) to couple normally to G protein and stimulate cAMP. Since arrestins both desensitize GPCRs and promote their internalization, interventions that decrease arrestin affinity for the V2R(R137H) should reestablish a more typical receptor phenotype. We have recently demonstrated using alanine substitution and C-tail truncation mutants of the wild-type V2R [the V2R(Ala6) and V2R(T362), respectively] that a single cluster of three serine residues in the tail of the V2R can substantially decrease the receptor's ability to bind βarrestin (11). Analogous mutants were constructed for the V2R(R137H), V2R(R137H,Ala6), and V2R(R137H,T362), with the anticipation that a decreased βarrestin affinity would normalize the receptor localization at the plasma membrane and correct its ability to stimulate adenylyl cyclase. Fig. 5*A*, *Upper*, shows that on live, unpermeabilized cells the HA-tagged V2R, V2R(R137H,Ala6), and V2R(R137H,T362) are easily observable at the plasma membrane by rhodamine fluorescence in the absence of agonist. The result contrasts with findings demonstrating relatively little antibody-labeled V2R(R137H) on the surface of unpermeabilized cells (Fig. 1). Addition of AVP to cells containing V2R, V2R(R137H,Ala6), and V2R(R137H,T362) results in the loss of plasma membrane fluorescence and the appearance of cytosolic fluorescence as a result of receptor endocytosis (Fig. 5*A*, *Lower*). The return of the plasma membrane population of V2R(R137H) tail mutants toward wild-type levels was confirmed by the binding of AVP (Fig. 5*B*). Thus, this suggests that the R137H mutation affects the steady-state distribution of trafficked receptor but not the trafficking *per se*.

A direct demonstration that the V2R(R137H,Ala6) and V2R(R137H,T362) receptor mutants have a lower affinity for βarrestin is provided in Fig. 6*A* using the βarrestin2-GFP fusion protein. In contrast to our findings for the V2R(R137H), the V2R(R137H,Ala6) and V2R(R137H,T362) are not constitutively associated with βarrestin as indicated by the homogenous distribution of βarrestin2-GFP in the cytoplasm of cells in the absence of hormone. Addition of AVP promotes βarrestin2-GFP redistribution to punctate areas at the plasma membrane but not redistribution with the receptor into endocytic vesicles. The inability of βarrestin2-GFP to remain associated and traffic with V2R in which the serine cluster has been removed has been previously observed and reflects decreased receptor affinity for arrestins (11).

The reduced ability of the V2R(R137H,Ala6) and V2R(R137H,T362) to bind βarrestins should improve their ability to stimulate cAMP if abnormal βarrestin-mediated desensitization contributes to the V2R(R137H) phenotype. Fig. 6*B* shows that the basal cAMP responses of all of the receptor variants are similar. Consistent with findings made in COS-7 cells (20), we observed a small agonist-mediated increase in cAMP over basal for the V2R(R137H). Mutation of the serine cluster in the V2R(R137H) C-tail resulted in a 6-fold enhancement in cAMP production of both the V2R(R137H,Ala6) and V2R(R137H,T362) mutants (Fig. 6*B*). These data suggest that the V2R(R137H) interacts with G protein, albeit less well than the wild-type V2R, and that its strong affinity for arrestin markedly inhibits this interaction.

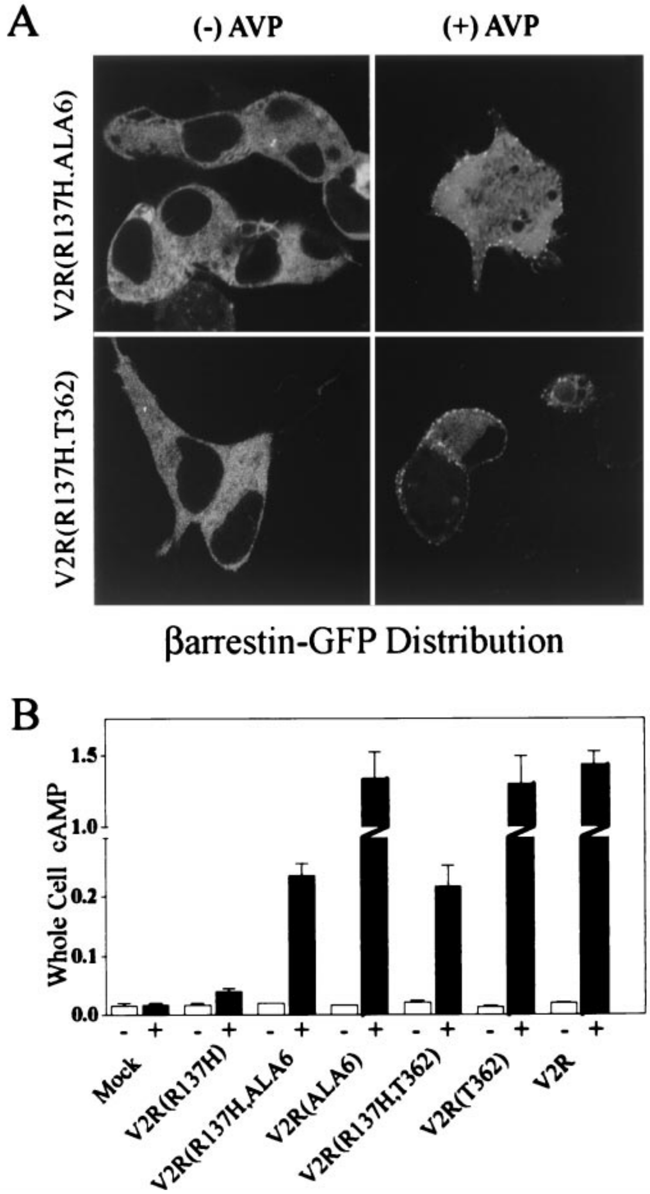

Fig. 6. βarrestin2-GFP translocation and adenylyl cyclase response of V2R, V2R(R137H,Ala6), and V2R(R137H,T362) in HEK-293 cells. (*A*) In the absence of agonist (*Left*), GFP fluorescence is cytosolic. Following exposure of the cells to 100 nM AVP for 30 min at 37°C, GFP fluorescence redistributes to punctate areas of plasma membrane (*Right*). (*B*) Cells were treated with vehicle or 2.5 μM AVP for 15 min, and whole cell cAMP was determined as described in *Experimental Procedures*. The absolute basal and stimulated cAMP responses were presented in units of (counts of [^{3}H]cAMP per min per well)/(counts of [^{3}H]adenine uptake per minute per well) and were mock (0.019 ± 0.009, 0.017 ± 0.006, n = 3); V2R(R137H) (0.018 ± 0.0045, 0.040 ± 0.008, n = 4); V2R(R137H,Ala6) (0.020 ± 0.0017, 0.23 ± 0.040, n = 4); V2R(Ala6) (0.017 ± 0.0006, 1.3 ± 0.31, n = 3); V2R(R137H,T362) (0.022 ± 0.0056, 0.22 ± 0.070, n = 4); V2R(T362) (0.015 ± 0.0021, 1.3 ± 0.34, n = 3); and V2R (0.021 ± 0.0024, 1.4 ± 0.19, n = 4). Data are expressed as the mean ± SD of three to four separate experiments.

Discussion

The pharmacological regulation of GPCR signal transduction by agonists, central to the treatment of many diseases, typically induces receptor desensitization. We observed that in the absence of agonist a naturally occurring, apparent "loss of function" V2R(R137H) mutant is phosphorylated, associated with βarrestin, and sequestered in intracellular vesicles; i.e., behaves as a constitutively desensitized receptor. These data provide a molecular basis for explaining the previous observations describing the uncoupling and agonist-independent internalization of this mutated receptor (18–20). Therefore, constitutive arrestin-mediated desensitization of GPCR signaling may represent an underlying and potentially unrecognized cause of loss of function mutations in GPCR-associated disorders.

GPCRs normally resensitize after dissociating from arrestins, and the distribution and number of GRK phosphorylation sites found in the GPCR tails are major determinants regulating arrestin trafficking behavior (11, 12). The GPCRs that form stable complexes with arrestins and that internalize together with them in endocytic vesicles commonly contain sequence motifs that can be efficiently phosphorylated (11, 13). In particular, the V2R contains a triplet serine (S359–361) whose removal impairs its association with arrestin and accelerates its resensitization (11). Removal of this triplet serine from the V2R(R137H) better enabled its Ala6 and T362 variants to activate second messenger and express at the plasma membrane. This suggests that the inability of plasma membrane-associated V2R(R137H) to activate G proteins in HEK-293 cells may be due in large part to a ligand-independent desensitization rather than an exclusive inability to activate G proteins.

The R137H mutation in the V2R DRH motif apparently disrupts the balance between desensitization and signaling to an extent that cannot be compensated *in vivo*. In contrast, other mutations affecting both processes might produce dissimilar or less extreme phenotypes. For example, the α1B-adrenergic receptor (αAR) belongs to a class of GPCRs that exhibit comparatively less cellular affinity than the V2R for arrestin, releasing it shortly after internalizing in clathrin-coated vesicles (11). In HEK-293 cells, the mutant αAR(A293E) binds βarrestin in the absence of agonist, but not well enough to prevent signaling (33). Rhodposin(K296E), isolated from a family with early onset retinitis pigmentosa (34), constitutively activates transducin *in vitro* (35). However, in mice the same mutant rhodopsin constitutively binds visual arrestin, does not signal, and results in retinal degeneration (34). Thus, the extent to which a given mutation affects receptor signal transduction depends upon the degree to which the mechanisms that generate and desensitize the signal are each affected.

Advances in the characterization of the cell biology of GPCR, GRK, and arrestin behavior should lead to other examples of abnormal desensitization producing receptor-related disease. For instance, elevated GRK expression has been associated with congestive heart failure and hypertension (36, 37), but a relationship between GRK expression and abnormal arrestin-mediated desensitization in these illnesses remains undetermined. Our study, indicating that inappropriate arrestin-mediated receptor desensitization may provide the etiology of a naturally occurring disease, suggests that molecular determinants of desensitization can be useful pharmacological targets.

This work was supported by National Institutes of Health Grants HL 61365 (to L.S.B.) and NS 19576 (to M.G.C.). S.A.L. is a recipient of a fellowship from the Medical Research Council of Canada. M.G.C. is an Investigator of the Howard Hughes Medical Institute and a recipient of an unrestricted Neuroscience Award from Bristol–Meyers Squibb.

1. Ferguson, S. S., Barak, L. S., Zhang, J. & Caron, M. G. (1996) *Can. J. Physiol. Pharmacol.* **74,** 1095–1110.
2. Krupnick, J. G. & Benovic, J. L. (1998) *Annu. Rev. Pharmacol. Toxicol.* **38,** 289–319.
3. Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G. & Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 17882–17890.
4. Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M. & Benovic, J. L. (1995) *J. Biol. Chem.* **270,** 720–731.
5. Barak, L. S., Ferguson, S. S., Zhang, J. & Caron, M. G. (1997) *J. Biol. Chem.* **272,** 27497–27500.
6. Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G. & Ferguson, S. S. (1999) *J. Biol. Chem.* **274,** 10999–11006.
7. Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G. & Ferguson, S. S. (1997) *J. Biol. Chem.* **272,** 27005–27014.
8. Menard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G. & Barak, L. S. (1997) *Mol. Pharmacol.* **51,** 800–808.
9. Ferguson, S. S., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A. M. & Caron, M. G. (1995) *J. Biol. Chem.* **270,** 24782–24789.
10. Barak, L. S., Warabi, K., Feng, X., Caron, M. G. & Kwatra, M. M. (1999) *J. Biol. Chem.* **274,** 7565–7569.
11. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S. & Caron, M. G. (1999) *J. Biol. Chem.* **274,** 32248–32257.
12. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G. & Barak, L. S. (2000) *J. Biol. Chem.* **275,** 17201–17210.
13. Innamorati, G., Sadeghi, H. M., Tran, N. T. & Birnbaumer, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2222–2226.
14. Birnbaumer, M. (1995) *J. Recept. Signal Transduct. Res.* **15,** 131–160.
15. Bichet, D. G. (1998) *Am. J. Med.* **105,** 431–442.
16. Schoneberg, T., Yun, J., Wenkert, D. & Wess, J. (1996) *EMBO J.* **15,** 1283–1291.
17. Morello, J. P., Salahpour, A., Laperriere, A., Bernier, V., Arthus, M. F., Lonergan, M., Petaja-Repo, U., Angers, S., Morin, D., Bichet, D. G. & Bouvier, M. (2000) *J. Clin. Invest.* **105,** 887–895.
18. Rosenthal, W., Antaramian, A., Gilbert, S. & Birnbaumer, M. (1993) *J. Biol. Chem.* **268,** 13030–13033.
19. Innamorati, G., Sadeghi, H., Eberle, A. N. & Birnbaumer, M. (1997) *J. Biol. Chem.* **272,** 2486–2492.
20. Schoneberg, T., Schulz, A., Biebermann, H., Gruters, A., Grimm, T., Hubschmann, K., Filler, G., Gudermann, T. & Schultz, G. (1998) *Hum. Mutat.* **12,** 196–205.
21. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J. & Sealfon, S. C. (1992) *DNA Cell Biol.* **11,** 1–20.
22. Shi, W., Sports, C. D., Raman, D., Shirakawa, S., Osawa, S. & Weiss, E. R. (1998) *Biochemistry* **37,** 4869–4874.
23. Barak, L. S., Ferguson, S. S., Zhang, J., Martenson, C., Meyer, T. & Caron, M. G. (1997) *Mol. Pharmacol.* **51,** 177–184.
24. Barak, L. S., Menard, L., Ferguson, S. S., Colapietro, A. M. & Caron, M. G. (1995) *Biochemistry* **34,** 15407–15414.
25. Sadeghi, H., Robertson, G. L., Bichet, D. G., Innamorati, G. & Birnbaumer, M. (1997) *Mol. Endocrinol.* **11,** 1806–1813.
26. Kallal, L. & Benovic, J. L. (2000) *Trends Pharmacol. Sci.* **21,** 175–180.
27. Pfeiffer, R., Kirsch, J. & Fahrenholz, F. (1998) *Exp. Cell Res.* **244,** 327–339.
28. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S. & Caron, M. G. (2000) *J. Biol. Chem.* **275,** 23120–23126.
29. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G. & Barak, L. S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3712–3717.
30. Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H. & Benovic, J. L. (1996) *Nature (London)* **383,** 447–450.
31. Sadeghi, H. & Birnbaumer, M. (1999) *Glycobiology* **9,** 731–737.
32. Sadeghi, H. M., Innamorati, G. & Birnbaumer, M. (1997) *J. Recept. Signal Transduct. Res.* **17,** 433–445.
33. Mhaouty-Kodja, S., Barak, L. S., Scheer, A., Abuin, L., Diviani, D., Caron, M. G. & Cotecchia, S. (1999) *Mol. Pharmacol.* **55,** 339–347.
34. Li, T., Franson, W. K., Gordon, J. W., Berson, E. L. & Dryja, T. P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3551–3555.
35. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A. & Oprian, D. D. (1992) *Neuron* **9,** 719–725.
36. Gros, R., Tan, C. M., Chorazyczewski, J., Kelvin, D. J., Benovic, J. L. & Feldman, R. D. (1999) *Clin Pharmacol. Ther.* **65,** 545–551.
37. Ungerer, M., Parruti, G., Bohm, M., Puzicha, M., DeBlasi, A., Erdmann, E. & Lohse, M. J. (1994) *Circ. Res.* **74,** 206–213.